are not used as a source of minerals to sustain the mammary supply during lactation. In other words, an osteoporotic skeleton is not an obligatory consequence of lactation in this species at least. Considerable difficulties exist in the determination of these requirements by the metabolic balance technique, since this method does not indicate when mobilization of minerals from the spongiosa is accompanied by mobilization from the shaft. Further, the balance over a whole lactation is often small in relation to the total quantities of materials analyzed and may be close to the analytical errors of the methods when the diet given is rich in minerals. The present observations may find some application in the determination of certain mineral requirements of lactation. Because the losses of Ca and P are unavoidable, involving the gradual liberation of reserves laid down at an earlier period, it has been difficult to estimate the requirements for lactation on the basis of the negative values obtained by the balance technique. Since the present study shows that, in the rat, it is possible to restrict the withdrawal of bone salt to the spongiosa, leaving the shaft unaffected, it seems reasonable to suggest that lactation requirements could be defined as those intakes which, for a given output of minerals in milk, will restrict the withdrawals to the spongiosa. Thus, using a similar technique, the problem of the mineral requirements for lactation in domestic animals might become more amenable to experimental attack. This preservation of the diaphyses may be an unattainable ideal in the high-producing dairy cow, but in sheep and swine it may be possible as in the rat. Also since Theiler (1934) pointed out that, in cattle, P deficiency had a more marked effect on the skeleton than Ca deficiency, the method might be more suitable for the determination of P than Ca requirement in the cow. While no evidence has yet been produced to show that a mild degree of osteoporosis is seriously detrimental to the individual, its avoidance would preserve the value of the skeleton from a structural standpoint.

SUMMARY

1. A technique for studying changes in the ends and shafts of the femur and tibia of the rat is described and used to follow the effect of gestation and lactation upon bone.

2. The percentage ash content and weight of dry, fat-free bones of well-fed rats were determined. During lactation and pregnancy the changes which occurred were confined to the bone ends.

3. The resorption of the spongiosa which occurred in lactating rats was not observed in non-lactating animals. The rate of replenishment of the depleted spongiosa at the end of lactation was the same, irrespective of whether the animals were then pregnant or non-pregnant.

4. The shafts of the bones of rats were little affected during lactation. The use of this finding in the determination of the Ca and P requirements of domestic animals is discussed.

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Qualitative Analysis of Proteins: a Partition Chromatographic Method Using Paper

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Gordon, Martin & Synge (1943*b*) attempted to separate amino-acids on a silica gel partition chromatogram, but found it impracticable owing to adsorption by the silica of various amino-acids. They obtained, however, good separations by using cellulose in the form of strips of filter paper. Following further work along these lines, the present paper describes a qualitative micro-analytical tech-

nique for proteins. Using only 200 μg. of wool, it is possible by this method to demonstrate the presence of all the amino-acids which have been shown to be there by other methods.

The method is rather similar to the 'capillary analysis' method of Schönbein and Goppelsroeder (reviewed by Rheinboldt, 1925) except that the separation depends on the differences in partition coefficient between the mobile phase and water-saturated cellulose, instead of differences in adsorption by the cellulose. That adsorption of the amino-acids by the cellulose plays no significant part is seen from Table 1, where the partition coefficient calculated from the rates of movement of the bands are compared with those found directly by England & Cohn (1935). Too much stress should not be laid upon the agreement of these figures, which are based upon an assumed water content of the saturated cellulose and the assumption that the ratio of the weight of *n*-butanol to paper is constant in all parts of the strip. This assumption does not hold accurately. Nevertheless, the conclusion seems justified that the cellulose is playing the role of an inert support.

The most satisfactory solvents are those which are partially miscible with water. Within a homologous series of solvents the corresponding rates of movement of the amino-acids change in the same direction as the water solubility of the solvent: solvents completely miscible with water can be employed provided that the water content is not too high. In this case, presumably, the cellulose, by a 'salting out' effect, allows the system to function as a partition chromatogram. However, the amino-acid bands obtained are much broader than is the case with immiscible solvents. This is no doubt due to a variation in the composition of the phases caused by the presence of the amino-acids. This effect has been noticed in the *n*-butanol-water system by England & Cohn (1935) and is the limiting factor in the amount of amino-acid that can be employed in a given chromatogram. It is reasonable to suppose that the phases will be more easily disturbed by employing a miscible rather than an immiscible solvent. The main effect of temperature on the rates of movement of amino-acids is also explicable in terms of the change in composition of the phases. Thus, in the phenol-water system, increase of temperature increases the miscibility and the rate of movement of the bands. In the collidine-water system the reverse is true. Further, the greater the difference between the working temperature and the critical solution temperature, the less sensitive will the rates be to change of temperature. However, though the absolute partition coefficients may be greatly changed, the ratios of the partition coefficients of the respective acids are almost unaltered.

There is an obvious advantage in working with unsubstituted amino-acids in that any substituent group, even though small, renders the physical properties of the derivatives more similar and hence increases the difficulties of separation. Martin & Synge (1941) and Gordon, Martin & Synge (1943*a*) failed to separate the slower moving acids when acetylated, whereas all the acids are separable by the present technique. Moreover, selective losses associated with acetylation and extraction are obviated.

A considerable number of solvents has been tried. The relative positions of the amino-acids in the developed chromatogram depend upon the solvent used. Hence by development first in one direction with one solvent followed by development in a direction at right angles with another solvent, amino-acids (e.g. a drop of protein hydrolysate) placed near the corner of a sheet of paper become distributed in a pattern across the sheet to give a two-dimensional chromatogram characteristic of the pair of solvents used. Advantage is taken of the colour reaction with ninhydrin (see Copley, 1941, for bibliography) to reveal the positions of the amino-acids.

Relation of partition coefficient to rate of movement of bands

The relation of the partition coefficient to the rate of movement of the band may be calculated by the method of Martin & Synge (1941).

A = cross-sectional area of paper + water + solvent,

A_L = cross-sectional area of solvent phase,

A_S = cross-sectional area of water phase,

α = partition coefficient

$$= \frac{\text{concentration in water phase}}{\text{concentration in solvent phase}},$$

$$R = \frac{A}{A_L + \alpha A_S}.$$

However, R is not conveniently measurable in paper chromatograms, so a new symbol, R_F is introduced.*

$$R_F = \frac{\text{movement of band}}{\text{movement of advancing front of liquid}} = \frac{RA_L}{A} = \frac{A_L}{A_L + \alpha A_S}$$

or
$$\alpha = \frac{A_L}{R_F A_S} - \frac{A_L}{A_S} = \frac{A_L}{A_S}\left(\frac{1}{R_F} - 1\right).$$

A_L/A_S is equal to the ratio of the volumes of solvent and water phase in the chromatogram.

* The symbol R_F is equivalent to the symbol R used by LeRosen (1942) who apparently received the paper of Martin & Synge (1941) too late to avoid using the symbol R in a different sense.

Table 1. *Partition coefficients calculated from R_F values*

No. of run ...	1	2	3	4	Direct measurements England & Cohn (1935)
% water in paper ...	28·7	18·0	22·6	17·7	
A_L/A_S ...	3·25	4·56	3·70	2·93	
Partition coefficients:					
Glycine	70·4	70·4	70·4	70·4	70·4
Alanine	35·9	39·9	43·7	36·6	42·3
Valine	12·2	14·1	14·8	12·5	13·8
*Nor*valine	8·7	10·8	10·5	9·2	9·5
Leucine	4·5	5·4	5·6	6·0	5·5
*Nor*leucine	3·5	4·2	4·4	4·6	3·2

Assuming a given water content of the paper, A_L/A_S may be deduced from the ratio of weight of dry paper to that of the developed chromatogram.

The water content of the paper is apt to vary from experiment to experiment and is difficult to measure in the presence of *n*-butanol or other solvent. Table 1 shows the partition coefficient calculated from the R_F and A_L/A_S values for four separate runs under slightly different conditions. The water content has been so chosen that the partition coefficient for glycine is equal to that given by England & Cohn (1935). The last column gives the direct measurements of England & Cohn.

The water content of saturated cellulose, according to the *International Critical Tables*, is 22 % on a dry-weight basis.

EXPERIMENTAL

The essentials of the apparatus consist of a filter-paper strip, the upper end of which is immersed in a trough containing the water-saturated solvent. The strip hangs in an airtight chamber in which is maintained an atmosphere saturated with water and solvent. The trough is provided with a bar over which the paper passes to prevent capillary siphoning between the outside of the trough and the paper. The apparatus is shown in Figs. 1–4.

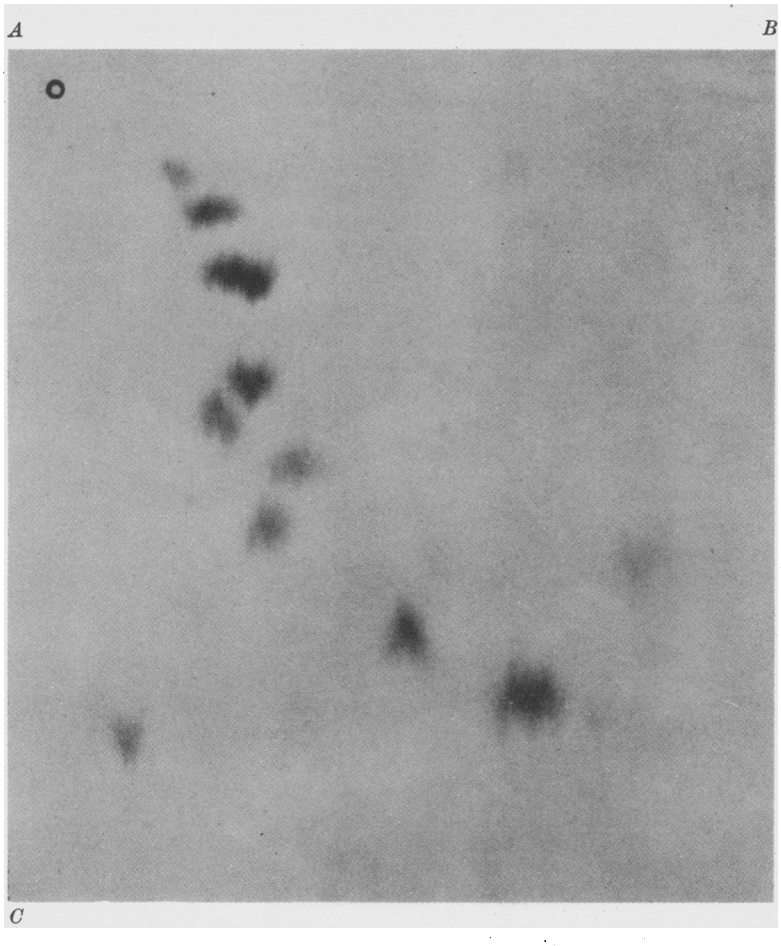
Chamber. For one-dimensional experiments, stoneware drain-pipes (6 or 9 in. in diameter and 2 ft. and 2 ft. 6 in. long respectively) have been found convenient. The pipe stands in a close-fitting tray hammered from a sheet of lead. The top of the pipe is ground flat and covered by a sheet of glass (Fig. 3). The glaze on these pipes is imperfect and a pipe should be reserved for each solvent and atmosphere. For two-dimensional experiments, the chamber consists of a glass-sided lead box, 30 × 30 × 5 in., made airtight with a well-fitting lead lid. A removable lead tray rests on the floor of the box. A completely air-tight tinplate box, using the tray as a water seal, was used when an atmosphere of coal gas was required.

Troughs. The troughs used in the one-dimensional experiments are constructed from ⅜ in. bore glass tubing, opened along its length by grinding (Figs. 1, 2). For two-dimensional work, the larger troughs required would be too fragile if of the same pattern. Therefore an iron frame supports the trough (of ½ in. bore thick-walled tubing) and carries strips of sheet glass which replace the bars. The cross-section is shown in Fig. 4.

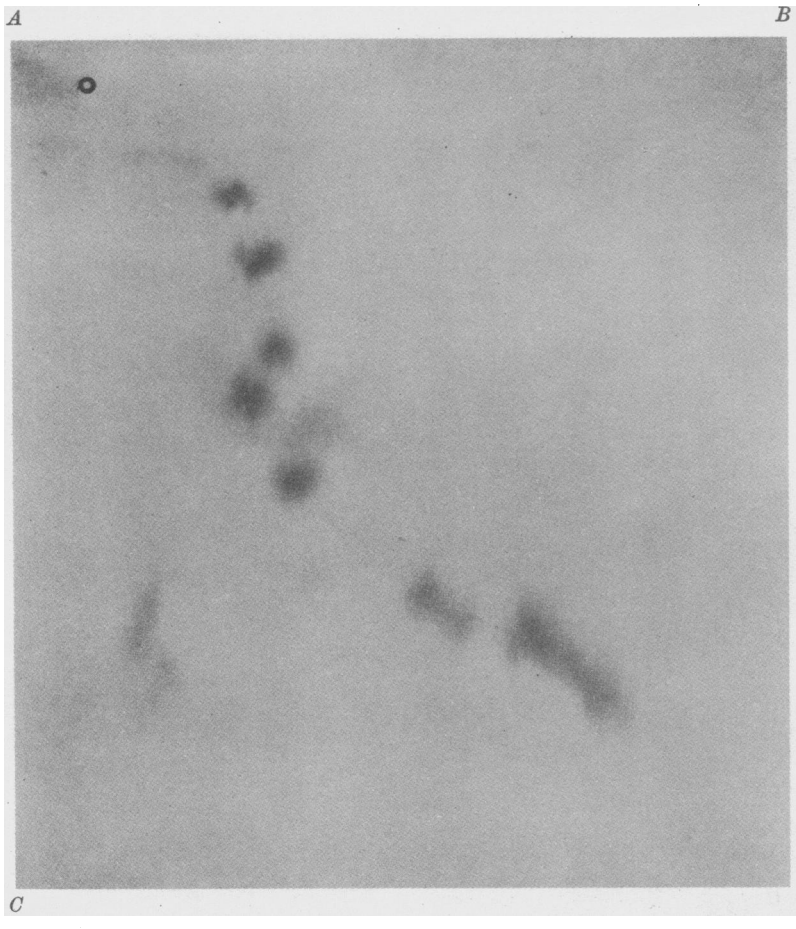
Paper. Whatman no. 1 filter paper has been used almost exclusively. Whatman no. 42, while giving satisfactory separations of amino-acids, was too dense to permit a convenient rate of flow of solvent. In an attempt to separate larger quantities of amino-acids, thicker papers have been tried. Of these, Whatman 'Accelerator' paper (⅓₀ in. thick) gave satisfactory separations but was too fragile to withstand normal handling. Whatman 'Seed Testing' paper (¹/₇₀ in. thick) was unsatisfactory as uniform flow of solvent could not be obtained. With most solvents the advancing front of liquid is coloured yellowish brown by material extracted from the paper. This contaminant can be partially removed by Soxhlet extraction with ethanol. Chromatographic washing is more effective. However, as the contaminant is so fast-running, relative to the amino-acids in most solvents (except phenol), it therefore does not interfere, and unextracted paper has usually been used.

Procedure

To run a one-dimensional chromatogram a strip of paper, 1·5 cm. or more in width and 20–56 cm. in length, is used. A pencil line is drawn across the strip about 5 cm. from one end. The solution, 2–4 μl. containing 5–15 μg. of each amino-acid to be analyzed, is applied along the centre portion of this line from the tip of a capillary tube. The end of the paper is fixed in the trough with a microscope slide. The trough and paper are now transferred to the chamber, which has been previously prepared by covering the bottom of the tray with a two-phase mixture of water and solvent to provide an atmosphere saturated with both components. The trough is filled with the water-saturated solvent and the lid put on the chamber. When the solvent has run a convenient distance (15–25 cm. in 6 hr.; 30–50 cm. in 24 hr., depending on solvent and temperature), the paper is removed and the position of the solvent front is marked. The strip is dried, either in an oven at 110° or by hanging in a drying cupboard through which hot air is sucked by a fan exhausting to the outside. After drying, the paper is sprayed with a solution of ninhydrin (0·1 % in *n*-butanol) and again dried. Finally, the paper is heated at 80° for 5 min. The bands are outlined in pencil, as fading of the colour takes place after a few days. When it is



Two-dimensional chromatogram of a wool hydrolysate (180 μg.) on Whatman no. 1 sheet. Hydrolysate applied at circle Run with collidine for 3 days in direction AB, then in direction AC with phenol for 27 hr. in an atmosphere of coa gas and NH_3 (produced from a 0·3% NH_3 solution). The filter employed in photographing renders the yellow prolin spot scarcely visible. (Photography by J. Manby, photographer to the University of Leeds.)



Two-dimensional chromatogram of a mixture of the hydrochlorides of 22 amino-acids (6–12 μg. of each) on Whatman no. 1 sheet, carried out as described under Pl. 1. As before, the yellow proline and orange hydroxyproline spots are not visible in the photograph. (Photography by J. Manby, photographer to the University of Leeds.)

desired to run a number of chromatograms simultaneously, the individual solutions may conveniently be placed side by side on a wide strip. It is seldom necessary to leave more than an interval of 1 cm. between the spots, but it is undesirable for the amino-acids to be too near the edge of the paper, as irregularities of flow are usually more pronounced there.

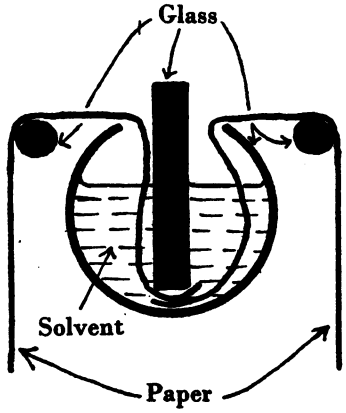


Fig. 1. Cross-section of small trough.

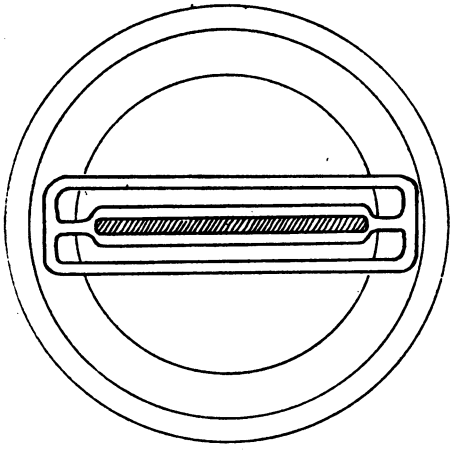


Fig. 2. Plan of small trough in pipe.

For two-dimensional analyses, a standard sheet $18 \times 22\frac{1}{2}$ in. is used (Pls. 1 and 2). The solution to be analyzed (6–12 μl., representing 200–400 μg. of protein) is placed near the corner, 6 cm. from either edge. The paper is held with one edge slightly overlapping the opening of the trough and pressed into it with a strip of sheet glass somewhat longer than the paper. After transfer to the chamber, prepared as above, the chromatogram is allowed to develop for 24–72 hr. The paper is then removed and dried in the drying cupboard, turned through a right angle, and returned to the trough. The next stage of development, again for 24–48 hr., now proceeds, the chamber, tray and trough having been prepared

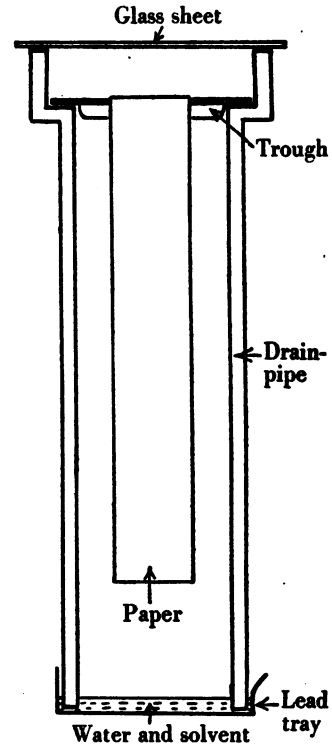


Fig. 3. Elevation of small trough in drain-pipe.

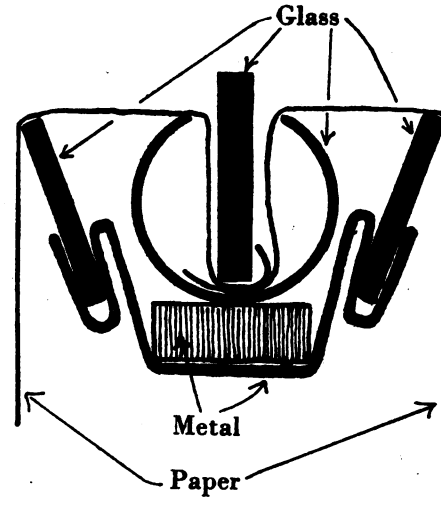


Fig. 4. Cross-section of large trough.

for the second solvent during the drying of the sheets. Subsequent treatment is the same as for the strips.

Table 2. *R_F values of amino-acid in various solvents on Whatman no. 1 paper, at room temperature*

Solvent ...	Phenol	Phenol	Phenol	Phenol	Phenol	Collidine	*n*-Butanol	*n*-Butanol	Tertiary amyl alcohol	Benzyl alcohol	1:1 *n*-Butanol benzyl alcohol	*o*-Cresol	*o*-Cresol	*m*-Cresol	*m*-Cresol	*p*-Cresol	*p*-Cresol	*Iso*-butyric acid
Addition ...	HCN	3% NH_3*	0·1% NH_3*	Coal gas	Cupron	—	Cupron	3% NH_3*	—	—	HCN	Cupron	Cupron + 0·1% NH_3*	Cupron	Cupron + 0·1% NH_3*	Cupron	Cupron + 0·1% NH_3*	—
Glycine	0·40 RP	0·40 RP	0·41	0·40	0·42	0·25 P	0·05	0·05	0·07 P	0·02	0·03 P	0·07	0·07	0·12 P	0·14 G	0·15 RP	0·19	0·36
Alanine	0·54 P	0·54 P	0·55	0·57	0·59	0·32 P	0·08	0·09	0·10 P	0·03	0·05 P	0·13	0·15	0·23 P	0·24 B	0·28	0·32	0·44
*Nor*valine	0·81 P	0·79 P	0·78	0·78	0·80	0·48 P	0·26	0·31	0·23 P	0·12	0·19 P	0·45	0·45	0·60 B	0·62 B	0·66 BP	0·66	0·71
Valine	0·77 P	0·76 P	0·76	0·78	0·77	0·45 P	0·20	0·22	0·18 BP	0·11	0·15 P	0·41	0·38	0·52 P	0·54 P	0·59 BP	0·60	0·65
*Nor*leucine	0·88 P	0·85 P	0·87	0·85	0·89†	0·60 P	0·47	0·51	0·42 P	0·27	0·36 P	0·72	0·67	0·80 B	0·78 P	0·82 BP	0·80	0·79
*Iso*leucine	0·86 P	0·81 P	0·87	0·82	0·86†	0·54 P	0·37	0·40	0·31 P	0·18	0·27 P	0·58	0·55	0·70 B	0·70 B	0·73 P	0·74	0·76
Leucine	0·85 P	0·83 P	0·86	0·84	0·88	0·58 P	0·38	0·46	0·36 P	0·21	0·31 P	0·61	0·62	0·73 B	0·73 B	0·73 BP	0·77	0·78
Phenylalanine	0·89 BP	0·87 P	0·90	0·86	0·93†	0·59 G	0·43	0·46	0·36 G	0·36	0·38 BG	0·81	0·74	0·82 GB	0·82 P	0·82 P	0·83	0·80
Tyrosine‡	0·64 GP	0·63 B	0·66	0·59	0·62	0·64 G	0·28	0·14	0·24 G	0·14	0·19 G	0·24	0·23	0·35 P	0·35 P	0·39 B	0·38	0·58
Serine	0·36 P	0·33 P	0·35	0·33	0·36	0·28 G	0·05	0·05	0·08 P	0·01	0·02 P	0·05	0·05	0·08 BP	0·08 B	0·11 P	0·13	0·34
Threonine	0·50 P	0·41 P	0·47	0·50	0·50	0·32 P	0·07	0·08	0·09 P	0·02	0·04 P	0·11	0·08	0·14 B	0·14 P	0·19 P	0·21	0·43
Hydroxy-proline	0·67 O	0·50 O	0·66	0·66	0·67	0·34 OY	0·07	0·05	0·10 O	0·04	0·05 O	0·27	0·23	0·32 GY	0·32 Y	0·40 BG	0·42	0·42
Proline	0·91 Y	0·85 Y	0·89	0·87	0·86†	0·35 Y	0·12	0·14	0·11 Y	0·12	0·12 Y	0·69	0·66	0·73 Y	0·75 Y	0·76 Y	0·78	0·57
Tryptophan	0·83 B	—	—	0·76	0·86	0·62 P	0·35	—	—	—	0·30 B	0·65	0·58	0·76	0·69	0·70 B	0·68	—
Histidine‡	0·69 RP	0·68 B	0·70	0·72	0·69	0·28 G	0·06	0·09	0·07 G	0·02	0·03 RG	0·25	0·33	0·34 P	0·44 GP	0·36 B	0·52	0·45
Arginine‡	0·59 P	0·89 P	0·85	0·67	0·62	0·16 P	0·03	0·05	0·02 P	0·01	0·01 P	0·02	0·44	0·07 P	0·76 P	0·12 BP	0·81	0·40
Ornithine‡	0·33 P	0·73 P	0·61	0·40	0·37	0·13 BG	0·01	0·03	0·02 P	0·00	0·00 P	0·01	0·21	0·03 P	0·47 P	0·04 P	0·59	0·24
Lysine‡	0·46 P	0·82 P	0·73	0·50	0·48	0·14 BG	0·01	0·03	0·02 P	0·00	0·01 P	0·01	0·20	0·04 P	0·54 P	0·07 BP	0·66	0·27
Aspartic acid ‖	0·15 BP	0·12 B	0·12	0·14	0·17	0·22 B	0·01	0·01	0·02 BP	0·00	0·00 P	0·00	0·00	0·01 RP	0·01 B	0·02 BP	0·02	0·31
Glutamic acid	0·25 P	0·13 P	0·19	0·24	0·28	0·25 P	0·01	0·01	0·02 P	0·00	0·01 P	0·01	0·01	0·01 P	0·02 B	0·03 BP	0·03	0·38
Lanthionine‡	0·20 RP	0·19 G	0·21	0·18	0·20	0·12 G	0·01	0·00	0·03 RG	0·00	0·00 P	0·01	0·01	0·01 R	0·03 RP	0·03 RP	0·05	0·21
Cystine‡	0·30 RP	0·24 YG	0·13§	0·13§	0·29	0·14 G	0·01	0·01	0·02 RG	0·00	0·00 P	0·02	0·01	0·04 P	0·04	0·02 RP	0·02	0·25
Methionine	0·80 P	0·76 P	0·83	0·82	0·81†	0·57 GP	0·26	0·05	0·27 P	0·17	0·21 P	0·58	0·49	0·64 B	0·63 P	0·71 BP	0·68	0·69

Colour given by ninhydrin = B, blue; G, grey; O, orange; P, purple; R, red; Y, yellow.

* The % figure refers to the strength of NH_3 solution present in quantity in the tray.
† These acids were applied to the paper after the solvent had already run some distance.
‡ These acids were put on as the hydrochlorides and neutralized with NH_3 before development.
§ Cystine is decomposed by these additions. R_F value refers to the decomposition product.
‖ Colour initially green. Only final colour shown in table.

Throughout the manipulations, care must be taken not to touch the paper with the hand as finger marks will show after heating with ninhydrin. Strips are handled with forceps, and sheets with special wide clips. For long runs, particularly overnight, it is desirable to lag the chamber, otherwise differences in temperature will cause water to distil from the tray, which may waterlog the paper and cause irregularity of flow.

When phenol is used, whether as first or second solvent, the faster moving bands are liable to distortion by the contaminant from the paper already mentioned. This trouble can be avoided by evenly spraying the top 5 in. of the strip or sheet with phenol before the trough is filled. In this way the contaminant is kept well ahead of even the fastest running amino-acids.

RESULTS

Rates of amino-acids in various solvents. R_F values for the most useful solvents are tabulated in Table 2. The colour given with ninhydrin is indicated by letters.

A number of other solvents has been tried and abandoned. Of these, ethyl acetate, methylethyl ketone, aniline, cyclohexanol, cyclohexanone, quinoline and 'light aniline fraction, b.p. 174–181°' were unsatisfactory as the amino-acids ran too slowly, whereas with methyl acetate, acetone, *sec.*-butanol, pyridine, the picolines and the lutidines, the bands were either too fast or unduly broadened.

or basic amino-acids would be more soluble in the water phase. In the alkaline pH range, the R_F values of aspartic and glutamic acids would thus decrease and those of arginine, lysine and histidine increase and vice versa in acid solutions (cf. Sharp, 1939). This is indeed found with NH_3 and HCl in phenol and in *n*-butanol, whereas dilute acid or base have very little influence on the monoamino-monocarboxylic acids. The effect of NH_3 in various solvents can be seen in Table 2.

Hydrolysates, or mixtures in which the ratio of soluble inorganic salts to amino-acid is high, give unsatisfactory chromatograms. The salt attracts water from the atmosphere and the solvent and causes local waterlogging of the paper. The amino-acids are not readily washed from these regions and the resulting bands are grossly distorted. This salt effect may be eliminated by impregnating the paper with salt and using the solvent and atmosphere equilibrated with saturated salt solution instead of with water. However, the colours given with ninhydrin are fainter and redder than those usually obtained and the rates are considerably slower.

In view of the alterations of rates with HCl, it is essential that the hydrolysate should be neutralized with NH_3 vapour after the hydrolysate has been applied to the paper, since some of the amino-acids are insoluble in neutral solutions.

To avoid the presence of excess salt, care must be taken to remove HCl from hydrolysates as far as possible, by repeated distillation *in vacuo*.

Table 3. *The effect of temperature on R_F values*

	Phenol					Phase composition % phenol in	
Temp.	Aspartic acid	Glutamic acid	Glycine	Lanthionine	Cystine	Phenol phase	Water phase
7– 8°	0·18	0·31	0·41	0·20	0·25	74·2	7·6
16–18°	0·22	0·34	0·44	0·17	0·28	72·7	8·1
20°	0·21	0·28	0·42	0·21	0·27	72·1	8·45
25°	0·19	0·33	0·45	0·23	0·28	71·05	8·75

Table 4. *The effect of temperature on R_F values*

	Collidine								Phase composition % collidine in	
Temp.	Aspartic acid	Glutamic acid	Glycine	Alanine	Valine	Leucine	Serine	Proline	Collidine phase	Water phase
10–15°	0·22	0·25	0·25	0·32	0·45	0·58	0·28	0·35	41·7 (10°) 49·0 (15°)	7·8 (10°) 5·0 (15°)
20°	0·04	0·04	0·06	0·10	0·27	0·37	0·10	0·12	54·9	3·4
25°	—	0·04	0·05	0·10	0·18	—	0·07	0·08	59·3	3·0

Effect of temperature. The variations of rates with temperature in phenol and collidine are shown in Tables 3 and 4. The phase compositions are also shown (data from *International Critical Tables*).

Additions of acids, bases and salts. It is to be expected that the ionized forms of the dicarboxylic

The effect of copper. In the early stages of this work we were puzzled by the occurrence of a pink band, running faster than, but not separated from, the main purple band. This was easily noticeable in the faster moving acids when benzyl alcohol, *tert.*-amyl alcohol, phenol, the cresols, *n*-butanol and

quinoline were used as solvents. These 'pink fronts' were, however, absent when collidine and *iso*butyric acid were used. It was found that Cu in the paper was responsible for this effect. Thus, impregnation with $CuSO_4$ solution or rubbing the paper with metallic Cu increased the 'pink front' at the expense of the main band. The Cu salt of glycine ran as an extended pink band (suggesting adsorption on the paper) with no purple glycine band, and with an R_F value corresponding to that of the 'pink front' of glycine. Further, the 'pink front' material was converted back to glycine by H_2S, HCN and NH_3. Neither acid nor NH_3 washing of the paper removed all the Cu, which was present also in commercial acid-washed paper. It was found that reagents which precipitate or form complexes with Cu prevented 'pink front' formation, e.g. H_2S, HCN, NH_3, or coal gas in the atmosphere, or pretreatment of paper with $K_4Fe(CN)_6$, or the addition of a freshly prepared solution of 'cupron' (α-benzoinoxime) to the solvent.

When phenol is used in an atmosphere containing NH_3, the Cu present in the paper catalyzes the formation of fast-running black material (Kohn & Fryer, 1893), which is liable to spoil the lower part of the chromatogram. However, those reagents which prevent 'pink front' formation will also prevent this catalysis. Since some ammonium salts give colours with ninhydrin, the use of H_2S with NH_3 is precluded. A dilute solution of 'cupron' in the phenol (0·1 %), or a trace of HCN, produced by the addition of a few crystals of KCN to the tray, have been found convenient methods. Coal gas, to be satisfactory, must be used in high concentrations.

DISCUSSION

The reproducibility of R_F values depends on the constancy of the following six factors: paper, temperature, quantity of amino-acid, extraneous substances, degree of saturation with water, supply of solvent and distance between starting-point and source of solvent. The irregularity of the band across the width of a wide paper affords a measure of the variations of the R_F values due to inhomogeneity of the paper. Thus for the valine band in phenol on Whatman no. 1 paper, a variation of ± 2 % has been found. By measuring from the 'centre of gravity' of a band or spot, this variation is largely eliminated. Papers of different densities will give widely different R_F values. Thus Whatman no. 42 gives much slower rates than Whatman no. 1. The chief cause of variation with temperature has already been discussed. With the solvents tested, changes in temperature do not cause large changes in rates except with collidine. The quantity of amino-acid has little effect on the R_F value though the size of the spot varies considerably. The rates of some of the amino-acids are seriously affected by acids and by reagents used to suppress the effect of Cu in the paper. The rate of a given amino-acid is slightly altered by the presence of another amino-acid. The importance of maintaining a fully saturated atmosphere was not at first appreciated; in Table 3 the scatter is partly due to an ill-fitting lid on the chamber. The paper may be undersaturated or oversaturated (waterlogged), according to the state of the atmosphere (cf. Table 1). Unlike a chromatogram in a tube, the ratio of solvent to stationary phase varies at different levels in a strip of paper. This ratio becomes progressively greater the shorter the distance from the trough. This characteristic distribution is disturbed both by an interruption of the flow of liquid and by the liquid accumulating at the bottom of the strip. In both cases, the relative rates of the amino-acids are slightly modified. As a result of the distribution of solvent, the greater the distance between the trough and starting-point of the amino-acid, the smaller the R_F value. However, this effect may be neglected for normal working distances.

When strict duplicates are run simultaneously in the same chamber, differences in R_F value do not exceed 4 %. Runs done at long intervals of time differ by much more than this, since close control of all the relevant factors has not been attempted, but relative rates are much more constant. Hence while it is not always possible to identify a given amino-acid by its R_F value alone, comparison with other amino-acids on the same strip allows this to be done with confidence; e.g. where a protein hydrolysate has been run with a benzyl alcohol-*n*-butanol mixture (1:1 by vol.) it is possible to recognize phenylalanine, leucine, *iso*leucine and methionine. Of the six factors discussed above, only the presence of extraneous substances will alter the order of amino-acid bands on the strip. In the case of two-dimensional chromatograms, even the presence of acid or base, while modifying the pattern, does not prevent the recognition of the amino-acids. Colour, of course, serves for the identification of several of the acids.

Resolution of two amino-acids becomes difficult if the R_F values do not differ by more than about 10 %. Thus leucine and *iso*leucine (R_F values 1·13:1) are not completely separable in benzyl alcohol.

In deciding which pair of solvents to use on a two-dimensional chromatogram, the R_F values of the amino-acids in one solvent are plotted as ordinates against those in the other solvent as abscissae. Fig. 5 shows the collidine and phenol-ammonia diagram. This is to be compared with the photographs (Pls. 1, 2) of the chromatograms of a wool hydrolysate and a mixture of the 22 amino-acids. The diagram is plotted for equal travel of the solvents but, in the sheets photographed, the colli-

dine has been allowed to travel much farther than the phenol in order to make full use of the size of the sheets.

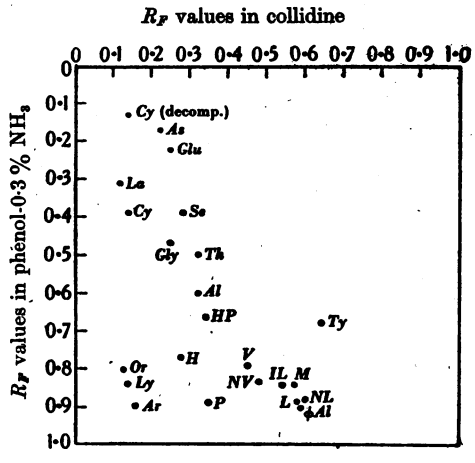


Fig. 5. Phenol-ammonia (0·3 %)/collidine diagram, showing expected positions of the amino-acids on a two-dimensional chromatogram prepared with these solvents. *Al*, alanine; *Ar*, arginine; *As*, aspartic acid; *Cy*, cystine; *Glu*, glutamic acid; *Gly*, glycine; *H*, histidine; *HP*, hydroxy-proline; *IL*, *iso*leucine; *La*, lanthionine; *L*, leucine; *Ly*, lysine; *M*, methionine; *NL*, *nor*leucine; *NV*, *nor*-valine; *Or*, ornithine; *φAl*, phenylalanine; *P*, proline; *Se*, serine; *Th*, threonine; *Tr*, tryptophan; *Ty*, tyrosine; *V*, valine.

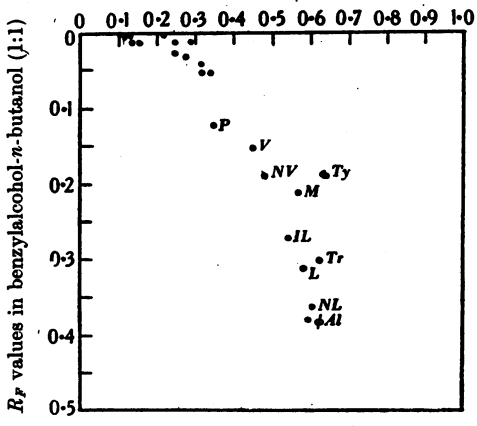


Fig. 6. Benzyl alcohol-*n*-butanol/collidine diagram, showing expected positions of the amino-acids on a two-dimensional chromatogram prepared with these solvents.

On the two-dimensional chromatogram, whatever the solvents, glycine and its straight chain homologues lie on a smooth curve. A branched chain results in slight deviation from this line, and a slowing of the rate. Since the presence of a hydroxyl group greatly decreases the R_F values in phenol, and has little effect on them in collidine, serine, threonine, tyrosine and hydroxyproline occur almost vertically above the corresponding unsubstituted amino-acids. Conversely, the bases are relatively slower in collidine than in phenol and thus occupy a position on the left of the diagram. It is of interest that proline, which has the same number of carbon atoms as valine, moves as fast as leucine in phenol and little faster than alanine in collidine; this behaviour is reminiscent of acetylproline, studied by Gordon *et al.* (1943*a*), which moved with acetylvaline or acetylalanine according to the solvent employed.

In Fig. 5, *iso*leucine, leucine, methionine and phenylalanine are crowded together, but by substituting *n*-butanol-benzyl alcohol mixture (1:1 v/v) for the phenol, a satisfactory separation of these components is obtained (see Fig. 6).

The small quantities of material employed by the method presented here are advantageous for many applications but make quantitative analysis difficult and preparative work impractical. Larger quantities can be handled in columns made from Whatman's 'Accelerator' paper, which can readily be reduced to powder, though advantage cannot then be taken of two-dimensional separation. Separations can be followed by periodic testing of a drop of effluent; material left in the column at any stage can be revealed by allowing a freshly prepared solution of ninhydrin (0·1 % in ether) to follow the solvent down the column. Coloured bands appear in a few hours at room temperature. For the weight of material handled these columns are bulky compared with those of silica gel. They have not as yet been investigated in detail but it is hoped that they will prove to be valuable. Columns of potato starch as used by Synge (1944) may prove to be superior.

The quantities of material employed in the two-dimensional technique are of the same order as those used in microbiological testing. Dr Happold kindly provided us with an opportunity of chromatographing a liver fraction containing growth factors for *C. diphtheriae*. Using a paper strip with *n*-butanol as solvent, at least six substances were shown to be present by treatment with ninhydrin. As in the case of the silica gel partition chromatograms, the paper chromatograms are by no means limited to the separations of amino-acids. Many bases and the NH_4 salts of many organic acids give colours with ninhydrin, and numerous colour reactions are available for other classes of compounds. The strip method can be employed to show the presence of more than one amino-acid in a given sample thought to be pure. The identity of a few micrograms of amino-acid can be established by running mixtures with authentic samples in a variety of solvents.

Any chromatographic method is well adapted for the discovery of further members of a known homo-

logous series, but the two-dimensional chromatogram is especially convenient, in that it shows at a glance information that can be gained otherwise only as the result of numerous experiments. The position of unknown substances on chromatograms developed with suitable pairs of solvents will also provide a clue to the existence of certain groups in the molecules, e.g. hydroxyl, acidic, basic or cyclic.

A few peptides have been shown to give satisfactory bands and also satisfactory colours with ninhydrin. In fact the work reported here was undertaken as a preliminary to the study of the products of the partial hydrolysis of proteins.

SUMMARY

1. A method of separating amino-acids on partition chromatograms by the use of water in cellulose (filter paper) as the stationary phase is described. Ninhydrin is used to reveal the amino-acids.

2. Phenol, collidine and *n*-butanol benzyl alcohol mixture (1:1 v/v) have proved useful as mobile phases. Other solvents have been investigated.

3. The partition coefficients calculated, normal water content of the paper being assumed, are close to those directly measured, showing that the cellulose acts as an inert support.

4. Two-dimensional chromatograms on sheets of filter paper are described; first one solvent is run in one direction, then, after the paper has been dried, another solvent is run in a direction at right angles to the first.

5. The presence of most of the amino-acids in wool, or in an artificial mixture of 22 amino-acids, can be demonstrated in a single experiment; all can be shown by suitable additional experiments. 200–400 μg. of protein are sufficient.

6. Hydroxy-amino-acids move more slowly than the corresponding amino-acids in phenol, but in collidine the rates are similar.

7. Ammonia selectively slows aspartic and glutamic acids and hastens the basic amino-acids. Acid has the reverse effect.

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Studies on Bacterial Amino-acid Decarboxylases

1. *l*(+)-LYSINE DECARBOXYLASE

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Amino-acid decarboxylases are produced by certain bacteria in response to growth in an acid environment (Gale, 1940, 1941), and it has been suggested that the neutralizing effect of the decarboxylation serves to protect the cell against the external acidity (Gale & Epps, 1942). The distribution of the decarboxylases among species and strains would indicate that each activity is produced by an enzyme specific for one amino-acid. The present communication deals with the extraction from the cell of *l*(+)-lysine decarboxylase, its partial purification, the properties of the partially purified enzyme and its resolution into the specific protein and a hitherto undescribed coenzyme. *l*(+)-Lysine decarboxylase is a component of the potential enzyme constitution of many of the coliform organisms, and two such organisms have been used as a source of enzyme in these studies. A preliminary note on the preparation of the specific enzyme and coenzyme has been published (Gale & Epps, 1943).